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### Document Version:

Publisher's PDF, also known as Version of record

### Citation for published version:

Salomon, Y, Gerst, J & Sole, J 1988, Regulation of melanotropin receptor function by calcium in the M2R melanoma cell line: Possible involvement of a calcium-binding protein. in F Bresciani (ed.), *Hormones and cancer 3: proceedings of the third International Congress*. Progress in Cancer Research and Therapy, vol. 35, pp. 164-167, Third International Congress on Hormones and Cancer, Hamburg, Germany, 1/9/87.

Total number of authors:

3

### Published In:

Hormones and cancer 3

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REGULATION OF MELANOTROPIN RECEPTOR FUNCTION BY CALCIUM IONS  
IN THE M2R MELANOMA CELL LINE: POSSIBLE INVOLVEMENT  
OF A CALCIUM-BINDING PROTEIN

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Expression of the melanotropin (MSH) receptor in melanoma cells is tightly coupled to the cell cycle and thus affords periodical regulation of certain cellular functions, notably melanin synthesis. The short-range minute-to-minute control of these cellular processes depends on circulating MSH concentrations and, as will be shown below, also on ambient levels of calcium.

Binding of MSH to the MSH receptor is the first and rate-limiting step in the sequence of biochemical events triggered in melanoma cells by this hormone (3,7). One of the established pathways activated by MSH in these cells is a GTP (G)-regulatory protein-mediated activation of adenylate cyclase (AC). In contrast to other hormones (e.g., biogenic amines, prostaglandins, peptide and glycoprotein hormones) that regulate AC in many cell types, we have shown that AC activation by MSH in M2R mouse melanoma cells uniquely requires extracellular calcium. The calcium dependency was seen at two concentration ranges saturating at  $\sim 10 \mu\text{M}$  and  $\sim 0.2 \text{ mM}$  (8,4). However, stimulation of AC in M2R cells either by prostaglandin  $\text{E}_1$  ( $\text{PGE}_1$ ) or forskolin was  $\text{Ca}^{++}$ -independent when measured in intact M2R cells, or in plasma membrane preparations derived from these cells. These results suggested that the  $\text{Ca}^{++}$ -dependent step in AC regulation must precede G-protein activation of the catalyst and is probably associated with regulation of the function of the hormone receptor itself. To further elucidate the role of  $\text{Ca}^{++}$  in MSH-receptor function, we investigated possible modulation by calcium of two known

functions of the (ii) interaction of [ $^{125}\text{I}$ ]-iodo-MSH (1) binding to M2R cells activation by MSH, concentration dependent previously found for kinetic analysis of increases the binding factor of  $\sim 20$  from super-saturating MSH can be demonstrated circulating physiological binding is absolute interaction of the determined by measurement dissociation from the is stable on the receptor is rapidly released manner;  $T_{1/2} = 2.6 \pm 0.4$   $T_{1/2} = 2.5 \pm 0.4$  min (n.s.) concluded that interaction with the G-protein, [ $^{125}\text{I}$ ]-MSH could also receptor by the at concentrations to  $< 10^{-10}$  to  $\sim 1 \text{ mM}$  resulted hormone. The specificity further assessed by other divalent cation binding, as supported following order:  $\text{Ca}^{++} > \text{Co}^{++} > \text{Cu}^{++}$   $\text{Ca}^{++}$  is likely to be MSH receptor regulation

The possibility that system may be modulatory protein with question by study antagonists, such as melittin (4,5), and of myosin light chain function. These peptides of  $\text{CaM}$ -regulated enzyme the ability of the (1,2). We first found membranes is selectively dependent manner ( $10^{-10}$  to  $100 \mu\text{M}$ , no effect

\*This peptide was kindly provided by Dr. Krebs of the University of

functions of the receptor: (i) hormone binding and (ii) interaction of the receptor with the G-protein. Using [<sup>125</sup>I]-iodo-MSH ([<sup>125</sup>I]-MSH), we established that hormone binding to M2R cells, the rate-limiting step in AC activation by MSH, was Ca<sup>2+</sup>-dependent and that the Ca<sup>2+</sup> concentration dependency of this process was similar to that previously found for AC activation by this hormone (4,8). Kinetic analysis of these results indicated that Ca<sup>2+</sup> increases the binding affinity of the receptor (K<sub>d</sub>) for MSH by a factor of ~20 from 418±40 nM to 23±3 nM MSH (n=3). Thus, at super-saturating MSH concentrations, i.e., >2x10<sup>-7</sup>M binding can be demonstrated even in the absence of calcium. However, at circulating physiological levels of MSH (≤10<sup>-9</sup>M), hormone binding is absolutely dependent on extracellular calcium. The interaction of the MSH receptor with the G-protein was determined by measuring GTP-dependent acceleration of [<sup>125</sup>I]-MSH dissociation from the receptor. While the tightly bound hormone is stable on the receptor for long periods of time (>30 min), it is rapidly released upon the addition of GTP in a dose-dependent manner; T<sub>0.5</sub>=2.6±0.2 min (n=3) in the presence of Ca<sup>2+</sup> and T<sub>0.5</sub>±2.5±0.4 min (n=3) in the absence of Ca<sup>2+</sup>. Thus, it was concluded that interaction of the receptor with MSH, but not with the G-protein, requires the presence of Ca<sup>2+</sup> ions. Bound [<sup>125</sup>I]-MSH could also be reversibly dissociated from the receptor by the addition of EGTA, which reduces ambient Ca<sup>2+</sup> concentrations to <<15 nM. Restoration of Ca<sup>2+</sup> concentration to ~1 mM resulted in rapid and complete rebinding of the hormone. The specificity of Ca<sup>2+</sup> in promoting MSH binding was further assessed by substitution of Ca<sup>2+</sup> with a series of other divalent cations. It was found that the efficiency of MSH binding, as supported by these cations, decreased in the following order: Ca<sup>2+</sup> >> Sr<sup>2+</sup> = Ni<sup>2+</sup> > Ba<sup>2+</sup> > Mn<sup>2+</sup> ≥ Cd<sup>2+</sup> > Co<sup>2+</sup> > Cu<sup>2+</sup> >> Mg<sup>2+</sup>. We thus concluded that Ca<sup>2+</sup> is likely to be the natural divalent cation involved in MSH receptor regulation.

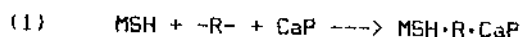
The possibility that the calcium sensitivity of this receptor system may be mediated by calmodulin (CaM) or a similar Ca<sup>2+</sup> modulatory protein was further investigated. We approached this question by studying the effects of various known CaM antagonists, such as fluphenazine, a bee venom-derived peptide - melittin (4,5), and a synthetic analog of the CaM-binding domain of myosin light chain kinase - M5\* (9,6) on MSH receptor function. These peptides are similar to the CaM-binding domains of CaM-regulated enzymes, the inhibition of which results from the ability of these antagonists to compete for available CaM (1,2). We first found that MSH-stimulated AC in M2R cell membranes is selectively inhibited by fluphenazine in a dose-dependent manner (IC<sub>50</sub>=16 μM). In the concentration range of 1-100 μM, no effect was observed on either basal or PGE<sub>1</sub>,

\*This peptide was kindly donated to us by Drs. Kennelly and Krebs of the University of Washington, Seattle, Wash., U.S.A.

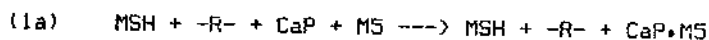
stimulation of AC. At higher concentrations ( $\geq 100 \mu\text{M}$ ), however, the drug inhibited both hormone-dependent and independent AC activity indiscriminately. Similarly, at low concentrations, melittin blocked MSH stimulation of M2R cell AC ( $\text{IC}_{50}=2.4 \mu\text{M}$ ), whereas inhibition of PGE<sub>1</sub> stimulation was achieved at substantially higher concentrations ( $\text{IC}_{50}>8 \mu\text{M}$ ) (5). The inhibitory effect of MSH-stimulated AC by MS was observed over the concentration range of 0.1–10  $\mu\text{M}$  ( $\text{IC}_{50}=1 \mu\text{M}$ ) (6). In comparison to fluphenazine and melittin, however, this peptide had absolutely no effect on either basal, PGE<sub>1</sub>, or GTP $\gamma$ S-stimulated AC, over the concentration range tested.

In parallel, and in agreement with these results, we also found that all three CaM antagonists inhibit <sup>125</sup>I-MSH-binding in a dose-dependent and non-competitive manner.  $\text{IC}_{50}$  values for fluphenazine, melittin, and MS inhibition of <sup>125</sup>I-MSH-binding were 14, 0.7 and 1.0  $\mu\text{M}$ , respectively. This result indicates that these inhibitors are not likely to compete with MSH for the hormone binding site on the receptor, nor do they bind to MSH itself.

We thus propose two alternative mechanisms for the observed inhibition of MSH receptor function by these CaM antagonists. In the first, hormone binding to the receptor R requires the association of the receptor with CaM or similar Ca<sup>2+</sup>-binding protein (CaP):

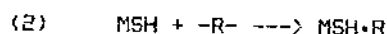


Inhibition of MSH binding to R will take place if the CaP binds the antagonist, a reaction that will prevent its association with the receptor and hence prevent activation of R as follows:

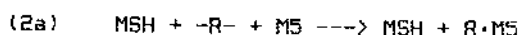


In this model, the function of R is inhibited by CaM antagonists in a manner similar to inhibition by MS of other CaM-regulated enzymes, i.e., by selective combination of these inhibitors with CaM.

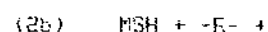
Alternatively, the antagonist may inhibit MSH-binding to R by directly associating with it. Receptor activation in this case will be described as follows:



where the MSH and MS binding sites on R are distinct and represented on the left (-R) and right (R-) side of R, respectively. Inhibition by CaM antagonists will consequently be formulated as:



or



or

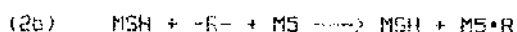


In this case, a CaM- the receptor itself) competitively inhibit predict competitive. These possibilities, analysis of the rest these antagonists mechanism described

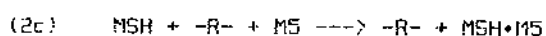
In summary, MSH to be uniquely and sensitive to inhibition have no conclusive pertinent site(s) in. Further studies are

The authors gratefully acknowledge the assistance of Rache grant to Y.S. from medizinische Forschungswissenschaft, F.R.G. Professor of Hormone

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or



In this case, a CaM-like sequence is expected to be present on the receptor itself and, if occupied by MS, will non-competitively inhibit MSH binding to R. Equations 2b and 2c predict competitive inhibition by the antagonist of MSH binding. These possibilities, however, can be eliminated, since kinetic analysis of the results showed that inhibition of MSH binding by these antagonists is non-competitive, in agreement with the mechanism described in equation 2a.

In summary, MSH receptor function has been demonstrated by us to be uniquely and unequivocally both calcium-dependent and sensitive to inhibition by CaM antagonists. Yet, at present, we have no conclusive proof to implicate CaM as the calcium-pertinent site(s) involved in melanotropin-receptor function. Further studies are still required to link these observations.

#### ACKNOWLEDGEMENT

The authors gratefully acknowledge the excellent secretarial assistance of Rachel Benjamin. This work was supported by a grant to Y.S. from the Hermann und Lilly Schilling-Stiftung für medizinische Forschung im Stifterverband für die Deutsche Wissenschaft, F.R.G. Y.S. is the Charles W. and Tillie K. Lubin Professor of Hormone Research.

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